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A Multigene Family Encoding a Diverse Array of Putative Pheromone Receptors in Mammals

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Summary

The vomeronasal organ of mammals is an olfactory sensory structure that detects pheromones. It contains two subsets of sensory neurons that differentially express $G\alpha_o$ and $G\alpha_{i2}$. By comparing gene expression in single neurons, we identified a novel multigene family that codes for a diverse array of candidate pheromone receptors (VRs) expressed by the $G\alpha_o^+$ subset. Different VRs are expressed by different neurons, but those neurons are interspersed, suggesting a distributed mode of sensory coding. Chromosome mapping experiments suggest an evolutionary connection between genes encoding VRs and receptors for volatile odorants. However, a dramatically different structure for VRs and the existence of variant VR mRNA forms indicate that there are diverse strategies to detect functionally distinct sensory stimuli.

Introduction

Pheromones are intraspecific chemical signals found throughout the animal kingdom. They regulate populations of animals by inducing innate behaviors and stereotyped changes in physiology (Karlson and Luscher, 1959; Wilson, 1963; Sorensen, 1996). Pheromones can serve as cues for overcrowding, impending danger, reproductive status, gender, or dominance. In rodents, a variety of pheromone effects have been reported. These include effects on estrus and the onset of puberty as well as the induction of mating and aggressive behaviors (Halpern, 1987; Wysocki and Meredith, 1987; Novotny et al., 1990; Singer, 1991).

The detection of pheromones is mediated by the olfactory system. However, sensory neurons that detect pheromones are typically segregated from those that detect volatile odorants (Keverne, 1983; Halpern, 1987; Wysocki and Meredith, 1987; Hildebrand and Shepherd, 1997). In mammals, sensory neurons in the nasal olfactory epithelium (OE) detect volatile odorants and some pheromones while those in an accessory olfactory organ, called the vomeronasal organ (VNO), are thought to be specialized to detect pheromones. The VNO is a tubular structure at the base of the nasal septum that is connected to the nasal cavity by a small duct. Signals from the OE are relayed through the olfactory bulb to the olfactory cortex and then to multiple brain regions, including those involved in conscious perception. In contrast, signals from the VNO are conveyed through the accessory olfactory bulb to the amygdala and hypothalamus, areas associated with the endocrine and behavioral responses induced by pheromones.

Volatile odorants are detected in the OE by as many as 1000 different types of odorant receptors (ORs), which are differentially expressed by olfactory sensory neurons (Buck and Axel, 1991; Levy et al., 1991; Nef et al., 1992; Strotmann et al., 1992; Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994). The ORs are thought to couple to the G protein α subunit $G\alpha_{olf}$ thereby initiating a cascade of transduction events that culminate in the generation of action potentials in the sensory axons (reviewed in Firestein, 1992; Reed, 1992; Ronnett and Snyder, 1992). Current evidence suggests that each OR may recognize a particular molecular feature that can be shared by many odorants (Ressler et al., 1994; Vassar et al., 1994; Axel, 1995; Buck, 1996; Mombaerts et al., 1996). This is consistent with a combinatorial coding model in which the identities of different odorants are encoded by different combinations of receptors, but each receptor serves as one component of the codes for many odorants.

By contrast, very little is known about how pheromones are detected or encoded in the VNO. Although VNO neurons (VNs) resemble olfactory sensory neurons in the nose, only a rare VN expresses an OR gene. VNs also lack a number of other olfactory sensory transduction molecules, including $G\alpha_{olf}$, which is highly expressed in olfactory neurons (Reed, 1992; Berghard et al., 1996; Wu et al., 1996). Instead, VNs express high levels of two other G protein α subunits, $G\alpha_o$ and $G\alpha_{i2}$ (Dulac and Axel, 1995; Halpern et al., 1995; Berghard and Buck, 1996). $G\alpha_o$ and $G\alpha_{i2}$ are expressed in spatially segregated subsets of VNs that form longitudinal zones in the VNO neuroepithelium. Interestingly, Dulac and Axel have identified a family of ~100 candidate pheromone receptors that appear to be expressed exclusively in the $G\alpha_{i2}$ subset (VNRs) (Dulac and Axel, 1995).

Here we describe a very different multigene family of ~140 members that codes for candidate pheromone receptors expressed by $G\alpha_o^+$ VNs (VRs). Each VR gene is expressed in only a small percentage of VNs. Moreover, neurons expressing different VR genes are interspersed throughout the $G\alpha_o^+$ zone of the VNO neuroepithelium. This patterning is similar to that previously seen for ORs and VNRs (Ressler et al., 1993; Vassar et al., 1993; Dulac and Axel, 1995) and is consistent with an ability of different neurons to detect different pheromones and with a distributed type of information coding in the VNO. Although VRs, like ORs and VNRs, are members of the G protein–coupled receptor (GPCR) superfamily, VRs have an unusual structure, which is quite different from that of ORs and VNRs and suggests a different mode of ligand recognition. This structural difference, together with the identification of variant forms of VR mRNAs and a chromosomal linkage between VR and OR genes, raises a number of questions about the evolution of VRs and the respective roles played by the three families of receptors in the detection of environmental chemicals.

Results

Cloning of a Gene Differentially Expressed in $G\alpha_o^+$ VNs

Different members of the OR and VNR families are expressed in different neurons in the OE and $G\alpha_{i2}^+$ zone

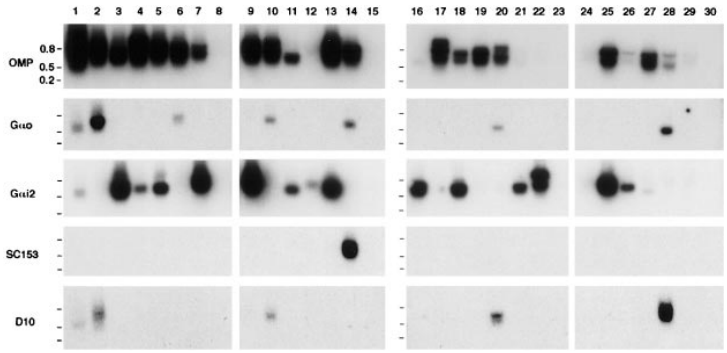

Figure 1. Southern Blot Analysis of cDNAs from Single VNO Neurons

Aliquots of amplified cDNAs from single VNs were size fractionated, blotted, and the blots were hybridized at 70°C to radiolabeled probes prepared from OMP, $G\alpha_o$, $G\alpha_{i2}$, or sc153 cDNAs or at 55°C to a D10 cDNA probe. Lanes 8, 15, 23, and 30 contain aliquots of PCR samples without cells. The size markers are indicated in kilobases. Note that most $G\alpha_o^+$ samples are $G\alpha_{i2}^-$ and vice versa and that all of the $D10^+$ samples are $G\alpha_o^+$.

of the VNO, respectively. It therefore appeared likely that the same would be true of sensory receptors expressed by $G\alpha_o^+$ VNs. The differential screening of cDNA libraries with cDNA probes prepared from a few neurons can be used to identify genes expressed in one neuron but not another (Buck et al., 1987). Using PCR, this can be accomplished with single cells (Brady and Iscove, 1993; Dulac and Axel, 1995).

To search for genes encoding receptors expressed by $G\alpha_o^+$ VNs, we looked for genes expressed in one $G\alpha_o^+$ VN but not another, using the PCR-based differential screening approach (see Experimental Procedures). In initial experiments, we isolated a series of mouse VNs, prepared cDNAs from the 3′ ends of mRNAs present in each, and amplified the single-cell cDNA fragments by PCR. Many of the amplified, single-cell cDNA samples hybridized to an OMP probe, confirming their derivation from VNs (Berghard et al., 1996) (Figure 1). With one exception, $G\alpha_o$ and $G\alpha_{i2}$ probes hybridized to different OMP^+ samples, allowing us to identify samples that were derived from $G\alpha_o^+$ VNs (Figure 1).

We next prepared a library from one of the $G\alpha_o^+$ single-cell cDNA samples (VN14) and isolated clones that hybridized to a probe prepared from VN14 but not to a probe prepared from another $G\alpha_o^+$ sample (VN2). We identified three $VN14^+VN2^-$ clones, which differed in size but were otherwise identical in sequence. None contained an open reading frame, which was not surprising, since in the method used the amplified cDNAs are only ~400 to 800 bp long and are derived from the 3′ ends of mRNAs (Brady and Iscove, 1993).

We next hybridized one of the $VN14^+VN2^-$ clones (sc153) to the original panel of single-cell cDNAs. sc153 hybridized to VN14 but not to any of the other cDNA samples (Figure 1). Consistent with this result, sc153 hybridized to only a small percentage (~0.3%) of VNs in VNO tissue sections (data not shown).

Using sc153 as probe, we were unable to identify a matching clone in a mouse VNO cDNA library. However, we were able to isolate an $sc153^+$ clone from a mouse genomic library that contained ~2 kb of DNA 5′ to the sc153 sequence. Using this 2 kb fragment as probe, we isolated a matching clone (D10) from the VNO cDNA library. Sequence analysis showed that sc153 and D10 were derived from the same gene, but that the D10 cDNA was truncated at the 3′ end and did not contain the final 685 bp of sequence present in sc153. Like sc153, D10 hybridized to only a small percentage of VNs in VNO tissue sections (data not shown).

The 5′ end of the D10 cDNA contained a short open reading frame, which encoded a protein fragment with homology to transmembrane domain 7 (TM7) of the Ca^{2+}-sensing receptor (CSR), a GPCR (Brown et al., 1993). When the TM7-related region of D10 (D10-TM7) was hybridized at reduced stringency (55°C) to the original panel of single-cell cDNAs, it labeled many of the $G\alpha_o^+$ samples but none of the $G\alpha_{i2}^+$ ones (except the one that was also $G\alpha_o^+$ and was probably derived from two cells) (Figure 1). Since D10 labeled only a small percentage of VNs in tissue sections under high stringency conditions, this suggested that many $G\alpha_o^+$ neurons express a gene related to D10 but not identical to it.

A Novel Multigene Family Encoding VNO Receptors

Hybridization of D10-TM7 to the VNO cDNA library at reduced stringency yielded a number of related cDNA clones (e.g., VR1–VR3; Figure 2). Additional related cDNAs were obtained by RT-PCR with degenerate primers (e.g., VR6 and VR7) or by screening the VNO cDNA library with a PCR product obtained from genomic DNA (e.g., VR4 and VR5) (see Experimental Procedures).

These cDNAs encode a novel family of proteins, which are members of the GPCR superfamily (Figure 2). Like other GPCRs, these VNO receptors (VRs) have seven hydrophobic stretches that may serve as membrane-spanning domains. Only 287 of 850 residues are identical in all of the molecules shown in Figure 2, indicating that the family is diverse. The VRs are related to two other types of GPCR, the CSR and the metabotropic glutamate receptors (mGluRs) (Tanabe et al., 1992; Brown et al., 1993). The most highly related molecule is the CSR; for example, VR1 is 31% identical to rat CSR (Riccardi et al., 1995), with the highest homology residing in the TM1–TM7 region (44%) (Figure 2). However, the VRs comprise a distinct family of receptors, which share novel sequence motifs and are more related to one another than they are to other receptors. For example, two divergent VRs, VR1 and VR4, are 70% identical in TM1–TM7 and 48% identical overall.

The VRs are unusual among GPCRs in having an extremely long N-terminal extracellular domain (Figures 2 and 7). This feature is shared by the CSR and mGluRs and by an unrelated class of GPCRs that includes several receptors for glycoprotein hormones (Segaloff and Ascoli, 1992). Importantly, the VRs are very different from both ORs and VNRs, which are also GPCRs (Buck and Axel, 1991; Dulac and Axel, 1995). VRs share none of the characteristic sequence motifs of ORs or VNRs.

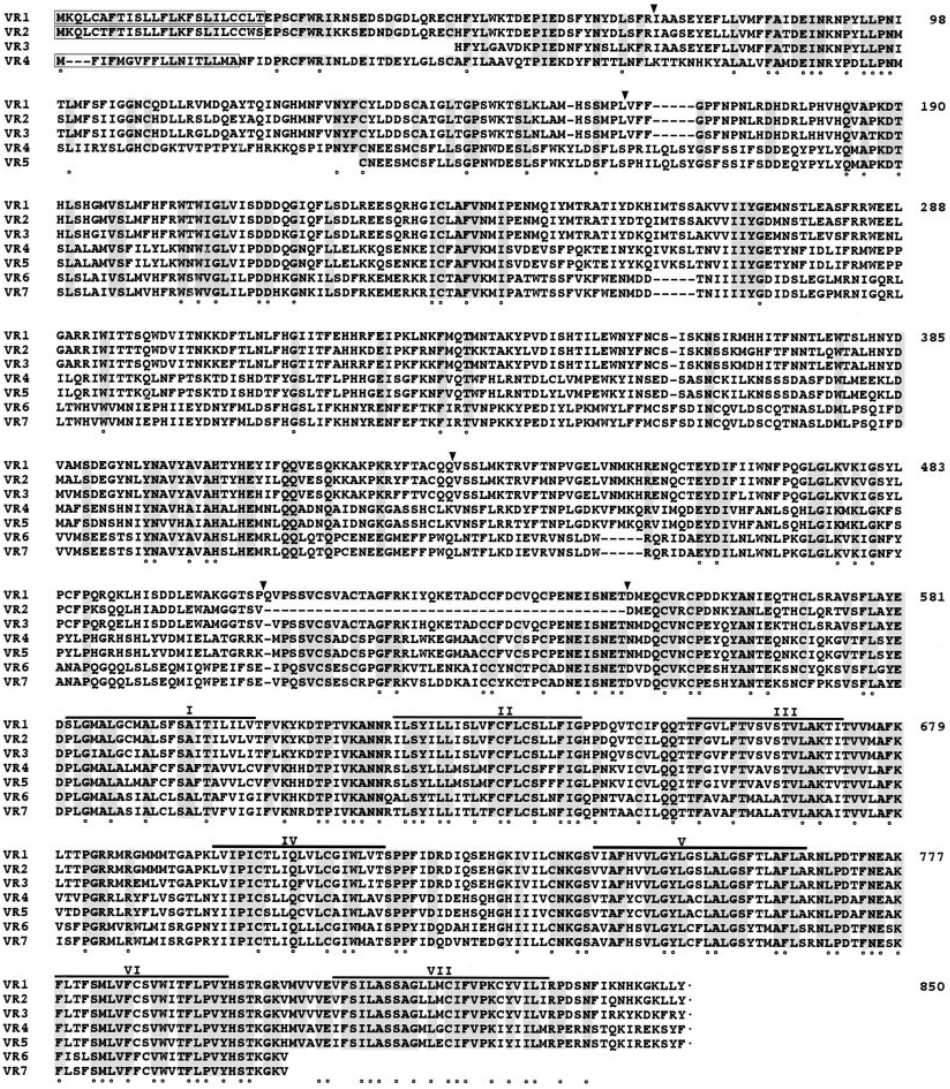

Figure 2. The Deduced Protein Sequences Encoded by VR cDNA Clones

Five cDNAs from a VNO cDNA library (VR1–VR5) and two obtained by RT-PCR from VNO RNA (VR6 and VR7) were sequenced, and the protein encoded by each was determined. Putative signal sequences are boxed, amino acids conserved in all of the proteins are shaded, and seven potential transmembrane domains are indicated by roman numerals. Open circles below the cDNA sequences indicate residues also found in the rat CSR (Riccardi et al., 1995), and arrowheads above the sequences indicate sites corresponding to exon–intron boundaries in the human CSR gene.

In addition, the size of the N-terminal extracellular domain of VRs (557 to 565 amino acids) far exceeds that of ORs and VNRs (~12 to 28 amino acids) (Figure 7). The VRs are most variable in the N-terminal domain (25% identical residues compared to 57% in TM1–TM7). In the structurally related mGluRs, the ligand-binding site is thought to reside in the large N-terminal domain (O'Hara et al., 1993; Takahashi et al., 1993). If this is also true of VRs, the accentuated diversity of the N-terminal domain may reflect an ability to recognize diverse pheromonal ligands.

Most of the VR cDNAs that we analyzed appeared to belong to one of three subfamilies of highly related molecules. For example, VR1, VR2, and VR3 are very similar, as are VR4 and VR5, and VR6 and VR7 (Figure 2). Nonetheless, our results indicate that all of these cDNAs were derived from different genes. First, all cDNAs were sequenced on both strands to rule out sequencing errors. Second, the RNA used for library construction and PCR came from an inbred mouse strain (C57BL/6J), so they cannot be allelic variants. Third, the error rates of reverse transcriptase (or Taq polymerase) cannot account for the extent to which the cDNAs differ. For example, VR4 and VR5 cDNAs are 99% identical in nucleotide sequence, but the reverse transcriptase used to prepare them has an error rate of only 3.6×10^{-5}/bp (Ji and Loeb, 1992).

Variant Forms of VR mRNA

Many of the VRs we characterized lacked a segment of the N-terminal domain present in other VRs. Invariably, the missing segment corresponded to a region of the

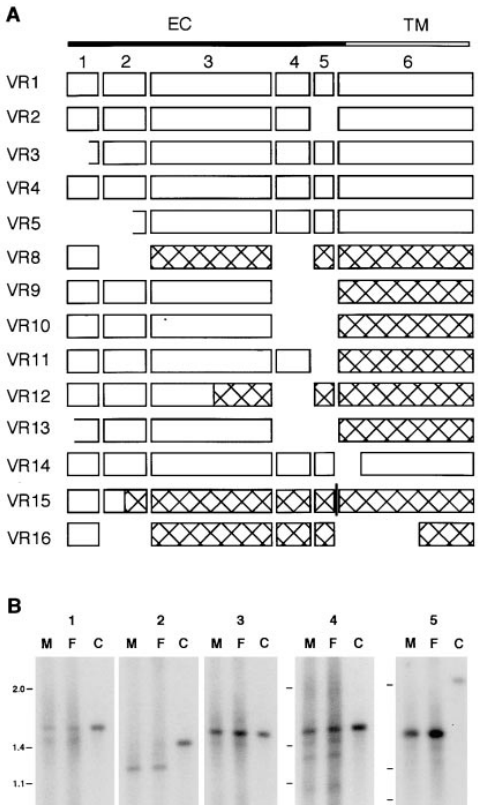

Figure 3. Variant VR mRNAs

(A) A schematic representation of different VR cDNA clones. The numbered boxes indicate corresponding exons 1 to 6 in the CSR gene. Empty boxes denote the open reading frame of each VR cDNA, and hatched boxes indicate coding region sequences that are untranslated due to a frameshift. The filled bar labeled EC indicates the proposed N-terminal extracellular domain, and the empty bar labeled TM indicates the region extending from the beginning of the first predicted transmembrane domain to the C terminus. Many of the cDNAs lack segments corresponding to individual exons in the CSR gene or contain additional, noncoding sequence at a putative exon–intron boundary (vertical bar in VR15), suggesting that different VR forms are generated by differential RNA splicing. In many cases, this results in a truncated protein that lacks the membrane spanning domains.

(B) Analysis of VR mRNAs in male versus female mice. Nested PCR was used to amplify sequences matching different VR cDNA clones, using as template cDNAs prepared from male (M) or female (F) VNO RNA or the cDNA clone (C). The PCR products were then size fractionated, blotted, and the blots hybridized to probes prepared from the different VR cDNA clones: (1) VR1, (2) VR2, (3) VR3, (4) VR4, (5) VR15. The positions of size markers are shown in kilobases. The results obtained with male and female cDNAs were similar. A major band corresponding in size to mRNA encoding a full-length receptor was seen in four of five cases. The short VR2 PCR product amplified from VNO cDNA encodes a truncated protein.

human CSR encoded by a single exon or pair of exons (Pollak et al., 1993) (Figures 2 and 3A). We also found several different VR cDNAs that contained a stretch of noncoding sequence at a site corresponding to a CSR exon–intron boundary (e.g., VR15) (Figure 3A). This suggested that the exon–intron structure of VR genes resembles that of the CSR gene and that variant forms of VR mRNAs might be generated by differential RNA splicing.

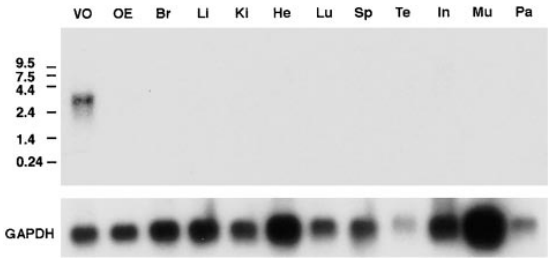

Figure 4. Northern Blot Analysis of VR mRNAs

One microgram of poly(A)$^+$ RNA from the mouse tissues indicated was size fractionated, blotted, and the blot was hybridized to a radiolabeled probe prepared from four VR cDNA clones (VR1, VR2, VR4, VR16). Control hybridization with a GAPDH probe (lower panel) confirmed the integrity of the RNAs used. RNAs: VNO (VO), olfactory epithelium (OE), brain (Br), liver (Li), kidney (Ki), heart (He), lung (Lu), spleen (Sp), and testis (Te), small intestine (In), skeletal muscle (Mu), and pancreas (Pa). The positions of size markers are indicated in kilobases.

Variant VR mRNAs could derive either from different genes or from the same gene by alternative RNA splicing. Consistent with the latter possibility, two pairs of cDNAs that we sequenced (VRs 8 and 9 and VRs 10 and 11) were identical in nucleotide sequence but were missing different segments (Figure 3A). However, when we used RT-PCR to amplify VNO mRNA sequences encoding five different VRs, we obtained one major PCR product in each case, regardless of whether the RNA used was from male or female mice (Figure 3B). In four cases, the size of the major product corresponded to a complete VR, even though one of the cDNAs (but not the PCR product) contained an intron (#5 [VR15]; Figure 3B). In one case, in which the cDNA lacked one exon (#2 [VR2]; Figure 3B), the major PCR product was even smaller and was found to lack two exons. Although PCR products of a smaller size were also seen in these experiments, they were much less abundant.

These results suggest that different VR forms derive from different genes. Thus, many VR genes may be expressed pseudogenes, which either lack one or more exons or have mutations that prevent proper RNA splicing. We cannot exclude the possibility that some variant VRs are functional, however. For example, some truncated VRs that lack transmembrane domains could conceivably be secreted pheromone-binding proteins.

Differential Expression of VR Genes in VNO Neurons

To investigate the tissue distribution of VR gene expression, we conducted Northern blot analyses in which size fractionated poly(A)$^+$ RNAs from different mouse tissues were hybridized to a mix of radiolabeled VR cDNAs (Figure 4). The mixed probe hybridized to VNO RNAs of ~1.9 to 3.7 kb, with intense hybridization to RNAs of 2.8 to 3.5 kb. It did not hybridize to RNAs from a variety of other tissues, including OE and brain. This suggested that VR genes may be expressed exclusively in the VNO.

We found two partial cDNAs that were highly related to VR cDNAs in the NCBI dbEST database, one from spleen and the other from two cell–stage mouse embryos. However, when we hybridized the most highly

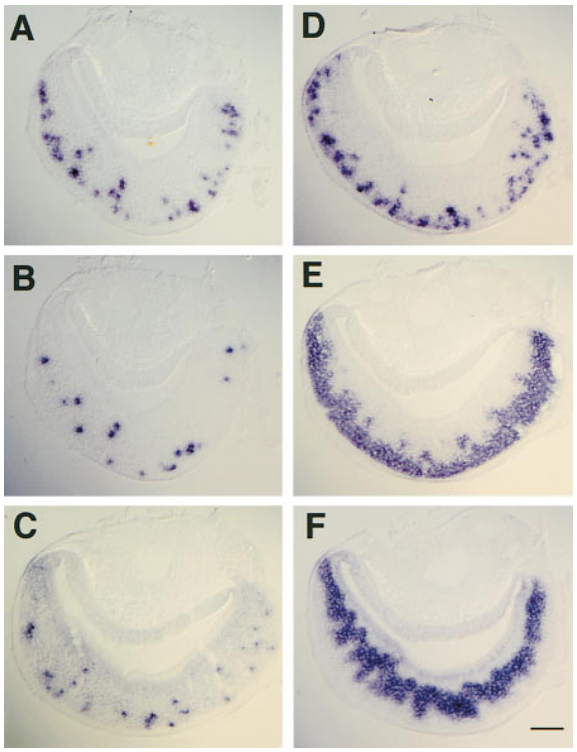

Figure 5. Analysis of VR Expression by In Situ Hybridization

Sequential coronal sections from a male mouse VNO were hybridized with digoxigenin-labeled probes prepared from a 3′ untranslated segment of the VR2 (A), VR3 (B), or VR15 (C) cDNA clone, a mix of these three probes (D), a $G\alpha_o$ probe (E), or a $G\alpha_{i2}$ probe (F). Each of the VR probes labeled a small percentage of neurons that appear to be confined to the $G\alpha_o^+$ zone of the VNO neuroepithelium but are scattered throughout that zone. The mixed VR probe labeled a larger percentage of neurons equivalent to the sum of the percentages labeled by its individual components. Similar patterns were seen in female mouse VNOs. Scale bar = 100 μm.

related VR cDNAs (VR6 and VR7) to spleen sections, only one questionably labeled cell was seen out of $\sim 1.4 \times 10^6$ cells with one VR probe, and none was seen with the other. The EST clones might be DNA contaminants or be due to the widespread but low level misexpression of tissue specific genes (Sarkar and Sommer, 1989); nonetheless, we cannot exclude the possibility that one or more VR genes are expressed at a low frequency in some other tissues.

To examine the patterns of expression of different VR genes in the VNO, we conducted in situ hybridization experiments (Figure 5). Labeled segments of the 3′ untranslated regions of three VR cDNAs were hybridized separately or in combination to sequential sections through the VNO. Probes prepared from $G\alpha_o$ and $G\alpha_{i2}$ cDNAs were hybridized to adjacent sections to delineate the $G\alpha_o^+$ and $G\alpha_{i2}^+$ zones of the VNO neuroepithelium.

The $G\alpha_o$ and $G\alpha_{i2}$ probes gave patterns of hybridization similar to those we had previously seen (Berghard and Buck, 1996). The $G\alpha_o$ probe hybridized to a wavy stripe of VNs in the basal (lower) region of the VNO neuroepithelium, whereas the $G\alpha_{i2}$ probe hybridized to an adjacent stripe of neurons in the apical (upper) part of the neuroepithelium (Figure 5). The waviness of the two zones appears to be caused by the periodic presence of blood vessels near the base of the epithelium (Berghard and Buck, 1996). Approximately 57% of VNs were labeled by the $G\alpha_{i2}$ probe, and 43% were labeled by the $G\alpha_o$ probe. The single layer of supporting cells located just beneath the epithelial surface was not labeled by either probe.

Each of the VR probes hybridized to a small percentage (2.4% to 5.7%) of VNs that appeared to be restricted to the basal, $G\alpha_o^+$ zone of the VNO neuroepithelium (Figure 5). Labeled neurons were scattered throughout the anterior–posterior and dorsal–ventral extent of the $G\alpha_o^+$ zone. Small clusters of labeled cells were sometimes seen, particularly with the VR2 probe. The mixed probe labeled a larger percentage of VNs (10.6%) that was almost equal to the sum of the percentages labeled by its individual components (10.8%) (Figure 5). Thus, different $G\alpha_o^+$ neurons must express different VRs.

No differences were seen in the patterns of hybridization obtained using VNOs from male and female mice, and no hybridization was observed in the nasal olfactory epithelium using either the mix of VR probes or a full-length VR cDNA probe (data not shown). Subsequent analyses of the size of the VR gene family and the number of VR genes recognized by the VR in situ hybridization probes allowed us to estimate the number of VR genes expressed by individual neurons (see below).

The Size of the VR Multigene Family

To investigate the size of the VR gene family, we hybridized several different mixed VR gene probes to a mouse genomic library, using high (70°C) or low (55°C) stringency conditions. A probe prepared from the membrane-spanning regions (putative exon 6) of several different cDNA clones hybridized to 59 and 98 clones per haploid genome equivalent, at high and low stringency, respectively. To obtain probes that were potentially more diverse, we amplified internal segments of putative exon 3 or 6 from genomic DNA by PCR with degenerate primers. At high stringency, these probes hybridized to 60 to 140 clones per haploid equivalent. These results indicate that there are as many as 140 VR genes in the mouse genome.

The VR probes that we used for in situ hybridization each labeled a small percentage of neurons. To determine how many VR genes each probe recognized, we hybridized probes prepared from the same VR cDNA segments to Southern blots of C57BL/6J mouse genomic DNA that had been digested with EcoRI or HindIII. Each probe hybridized to a small number of restriction fragments (Figure 6). Given the small size of the probes ($\sim$350 to 450 bp), most of these fragments should represent at least one gene, provided that there are no introns in the region probed. Consistent with this assumption, the VR2 probe hybridized to seven different restriction fragments, as many as five of which could be accounted for by characterized VR cDNAs that were 91% to 98% identical to VR2 in the region probed.

Given the number of genes recognized by each VR probe and the percentage of $G\alpha_o^+$ neurons that hybridized to each, we estimate that each VR gene may be expressed in only $\sim$1.1% to 1.9% of $G\alpha_o^+$ VNs. Since

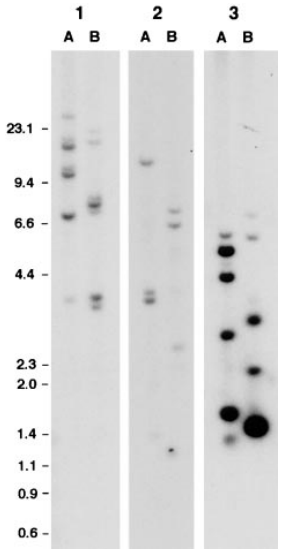

Figure 6. Genomic Southern Blot Analysis of VR Genes

Five micrograms of mouse liver DNA was digested with EcoRI (A) or HindIII (B), size-fractionated, blotted, and the blot was hybridized with radiolabeled probes prepared from 3′ untranslated segments of the VR2 (1), VR3 (2), VR15 (3), or VR4 cDNA [not shown, but identical to (3)]. The positions of size markers are shown in kilobases.

there appear to be 60 to 140 VR genes in the mouse genome, this suggests that each $G\alpha_o^+$ VN may express only one, or at most a few, VR genes.

Linkage of Chromosomal Clusters of VR and OR Genes

We previously found that there are clusters of OR genes at multiple chromosomal sites in the mouse genome (Sullivan et al., 1996). To investigate the chromosomal locations of VR genes, we used the Jackson Laboratory backcross DNA mapping panel, which allows the mapping of mouse genes using interspecies mouse crosses.

Probes prepared from the 3′ untranslated regions of VR2 and VR4 cDNAs were first hybridized to Southern blots of genomic DNAs from two mouse species, C57BL/6J and Mus spretus, which had been digested with different restriction enzymes. EcoRI digests showed a number of restriction length polymorphisms with both VR probes. The VR probes were then hybridized to EcoRI-digested DNAs from a large panel of different backcross mice ((C57BL/6J × M. spretus) × M. spretus).

The patterns of inheritance of the polymorphic fragments recognized by the two VR probes (data not shown) allowed us to assign chromosomal locations to approximately nine VR genes. Using the VR4 probe, we could follow the inheritance of four polymorphic restriction fragments. All of these cosegregated in the backcrosses and mapped to the proximal end of chromosome 7 (near *D7Bir5*). Five restriction fragments were followed for the VR2 probe. Again, all of the restriction fragments cosegregated, allowing us to map the VR2 fragments to the distal end of chromosome 4 (near *D4Bir1*). Given the resolution of the genetic mapping, the cosegregating fragments can be no more than 3.8 cM from one another. These results indicate that VR genes are located near the ends of at least two different mouse chromosomes. They also indicate that highly related VR genes are clustered at the same chromosomal locus, as previously seen for OR genes in our studies and others (Ben-Arie et al., 1994).

The VR4 gene subfamily appears to be closely linked to one OR gene locus, *olfR5* (Sullivan et al., 1996). Although the VRs and ORs were mapped in different mouse crosses, the synaptotagmin-3 gene (*Syt3*) was mapped in both crosses, allowing an estimate of their relative positions. The OR locus mapped 15.05 cM proximal to *Syt3* while the VR4 gene cluster mapped 14.89 cM proximal to *Syt3* (Jackson Laboratory Mouse Genome Informatics), suggesting a close linkage between VR and OR genes at the proximal end of chromosome 7. Our previous studies indicate that multiple OR gene loci arose via a series of duplications of very large chromosomal domains that maintained linkages between OR genes and members of other gene families (Sullivan et al., 1996). These results therefore suggest that VR genes and OR genes might have been linked in a primitive ancestor. They also suggest the possibility that additional clusters of VR genes might be linked to other OR gene loci.

Discussion

Our results define a novel multigene family that codes for candidate pheromone receptors in the mouse VNO. The family contains as many as 140 different genes, which are differentially expressed by sensory neurons in the $G\alpha_o^+$ zone of the VNO neuroepithelium. The VRs encoded by this family are dramatically different in structure from the two previously identified classes of receptors expressed in the olfactory system, the ORs and VNRs. This difference, together with the finding of variant forms of VRs, raises a number of intriguing questions about the strategies used to sense pheromones versus volatile odorants.

The VRs, an Unusual Family of Candidate Pheromone Receptors

The VRs are members of the large GPCR superfamily. In this respect, they resemble ORs in the nose and VNRs in the $G\alpha_{i2}^+$ zone of the VNO (Buck and Axel, 1991; Dulac and Axel, 1995), as well as a variety of receptors expressed by chemosensory neurons in the nematode worm, Caenorhabditis elegans (Troemel et al., 1995; Sengupta et al., 1996). However, the VRs comprise a distinct receptor family in mouse. Simultaneous studies in rat have revealed a similar receptor family in that species as well (see accompanying paper, Herrada and Dulac, 1997 [this issue of *Cell*]). Unlike these receptors and most other GPCRs, the VRs have an unusually long N-terminal extracellular domain (Figure 7). This unusual feature is shared by two other types of GPCR that are related in sequence to VRs, the CSR and the metabotropic glutamate receptors (Tanabe et al., 1992; Brown et al., 1993).

Structure–function studies employing mutagenesis techniques have suggested that many GPCRs with short N-termini (like ORs and VNRs) may bind to ligand in a

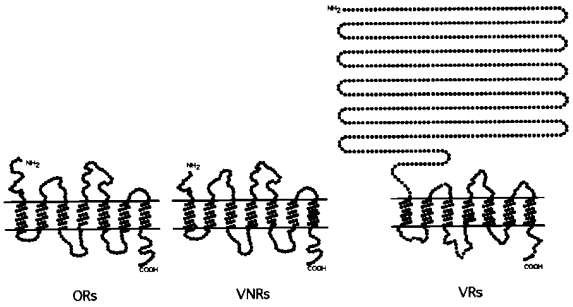

Figure 7. A Schematic Comparison of ORs, VNRs, and VRs

Schematic diagrams are shown of the predicted structures of odorant receptors (ORs), candidate pheromone receptors expressed in $G\alpha_{i2}^+$ VNO neurons (VNRs), and the candidate pheromone receptors expressed in $G\alpha_o^+$ VNO neurons (VRs). Balls indicate individual residues, and horizontal bars denote the limits of the plasma membrane. Like other G protein–coupled receptors, ORs, VNRs, and VRs all have seven potential membrane-spanning domains. However, VRs have an extremely long N-terminal extracellular domain compared to ORs and VNRs. In VRs, this domain may interact with ligand, whereas in ORs and VNRs, ligand binding may occur in a ligand-binding pocket formed by the transmembrane domains.

pocket that is formed in the plane of the membrane by a combination of the transmembrane domains (Strader et al., 1995). Extracellular loops between the transmembrane domains may also contribute to ligand binding, at least in some cases. In contrast, in the mGluRs, it appears that the large N-terminal extracellular domain plays an important role in ligand binding or may mediate it entirely (O'Hara et al., 1993; Takahashi et al., 1993). Given their structural resemblance to mGluRs, this may also be the case for VRs. If so, the extensive diversity seen in the N-terminal domains of VRs may reflect an ability to recognize different pheromones.

A structural resemblance has been proposed between the mGluRs and a variety of nutrient-binding proteins in bacteria (O'Hara et al., 1993; Quiocho and Ledvina, 1996). The bacterial proteins appear to bind ligand in a cleft between two globular domains, inducing an allosteric change that encloses the ligand (Quiocho and Ledvina, 1996). A similar "venus flytrap" design has been suggested for mGluRs, except that here an allosteric change in the N-terminal domain might be translated into G-protein coupling via intracellular domains (O'Hara et al., 1993).

Another unusual feature of the VR family is the existence of variant forms of VR mRNAs, which lack segments that are likely to derive from individual exons. Our studies suggest that different VR mRNA forms are derived from different VR genes rather than from the same gene by alternative RNA splicing. Some VR genes may be expressed pseudogenes, which lack one or more exons, or have mutations that prevent proper splicing. A less likely possibility, but one that cannot be excluded, is that the splicing machinery is regulated by neuronal activity and that exposure to ligand promotes the generation of either normal VRs or variants.

Some of the variant VR mRNAs encode a receptor that lacks a short segment of the N-terminal extracellular domain, while others encode a truncated protein lacking transmembrane domains. The variant proteins may very well be unstable or nonfunctional. However, we cannot rule out the possibility that they function as receptors with altered ligand-binding properties or as secreted proteins that bind pheromones.

Information Coding in the VNO

Our studies suggest that each $G\alpha_o^+$ VN expresses only one, or at most a few, VR genes. Thus, the information that each neuron transmits to the brain may reflect the specificity of only one VR. Neurons that express different VR genes are interspersed in the $G\alpha_o^+$ zone of the VNO neuroepithelium, further suggesting that sensory stimuli are encoded in a distributed fashion in the $G\alpha_o^+$ zone of the VNO. This arrangement resembles that previously seen for ORs and VNRs. Thus, the basic strategy used to organize and encode sensory stimuli at the level of the sensory epithelium appears to be conserved in the main and accessory olfactory systems.

The mechanisms that generate a distributed pattern of receptor expression in the OE and VNO may also be similar but are presently unknown. In the case of ORs, it has been suggested that the developing neuron randomly selects one gene for expression from among the members of a large receptor gene set (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994). This may also be true for VRs, although the presence of small clusters of $VR2^+$ cells suggests the possibility of an additional layer of regulation.

Our experiments indicate that each $G\alpha_o^+$ VN may express only one, or at most a few, VR genes. However, many VR genes may give rise to variant, nonfunctional VRs. Thus, many neurons either lack functional VRs or express one functional VR along with one or more nonfunctional ones. A precedent for this is seen in immunoglobulin heavy-chain gene expression in lymphocytes. Here, the expression of a functional protein from one rearranged allele prevents rearrangement and expression of the second allele (Schatz et al., 1992). A similar feedback mechanism could operate in VNs: the cell might randomly activate one VR gene after another until a functional VR is expressed.

VRs and VNRs are expressed in spatially segregated zones of the VNO neuroepithelium, which anatomical studies indicate project axons to different parts of the accessory olfactory bulb (Jia and Halpern, 1996). This raises the possibility that information provided by VRs and VNRs remains segregated throughout the vomeronasal pathway and is ultimately targeted to different regions of the amygdala or hypothalamus that mediate different effects of pheromones.

Different Strategies to Detect Pheromones and Volatile Odorants

In the mouse, there are as many as 1000 different ORs, 100 VNRs, and 140 VRs. The ability of the OR repertoire to discriminate thousands or tens of thousands of different volatile odorants suggests that ORs could also encode the identities of different pheromones. Why does the olfactory system employ different receptors in the VNO and OE?

One possible explanation is that the different receptors are uniquely suited to the distinct functions they

subserve: the discrimination of a multitude of volatile odorants versus the generation of innate responses to pheromones. While ORs might recognize structural features common to many odorants, VNO receptors might, instead, be highly specific for particular pheromones, thereby preventing innate responses to inappropriate stimuli. VNO receptors might also "corecognize" a pheromone and a specific carrier protein that binds to the pheromone. The carrier might "tag" the pheromone as being derived from an appropriate source or narrow the spectrum of molecules that can be recognized by imposing an additional requirement that they interact with a second protein (Vogt, 1987; Singer, 1991; Buck, 1995; Hildebrand and Shepherd, 1997).

The VNO neuroepithelium shares morphological and biochemical features and a common embryologic origin with the OE. This suggests that in a primitive ancestor, VRs and ORs might have been expressed in the same sensory epithelium, perhaps even in the same cell. Our experiments show that at least one cluster of VR genes is linked to an OR gene locus in the mouse genome. Thus, primitive clusters of VR and OR genes might also have been linked, perhaps permitting the coordinate regulation of these genes either in the same sensory epithelium or in the same cell.

Another question raised by our studies is why there are two distinct families of VRs expressed in the VNO. As already discussed, the VRs and VNRs have very different structures and could have different modes of ligand recognition. It is therefore conceivable that VRs and VNRs recognize different structural classes of pheromones, or that one family corecognizes carrier proteins that the other does not.

Having a remote ligand-binding site in the N-terminal domain may also allow VRs to evolve more rapidly to accommodate the recognition of new pheromones that arise during the formation of new species, thereby aiding in speciation. The existence of variant VR mRNAs may reflect such a rapid evolution. Consistent with this notion, in preliminary experiments, we have obtained evidence in several vertebrate species, including human and frog (Xenopus laevis), for families of genes that are related to mouse VR genes but are distinctly different. However, the functions of these genes remain to be explored.

Experimental Procedures

Preparation and Analysis of Single-Cell cDNAs

Male mouse (C57BL/6J) VNOs were minced, incubated in Trypsin-EDTA (GIBCO), and triturated to obtain dissociated cells. The cells were centrifuged (1000 rpm, 5 min) and resuspended in phosphate buffered saline plus 0.1% bovine serum albumin. Individual cells that appeared to be neurons were transferred to separate tubes with a microcapillary pipet.

cDNAs were prepared from each cell and amplified according to Brady and Iscove (1993) and Dulac and Axel (1995), with minor modifications. In brief, cDNAs were prepared from the 3′ ends of mRNAs by reverse transcription with an oligo(dT) primer, and a poly(dA) stretch was added to each cDNA with terminal transferase. The cDNAs were then amplified by PCR with one of two primers, AL1 (ATTGGATCCAGGCCGCTCTGGACAAAATATGAATTC(T)24) (Dulac and Axel, 1995) or AL3 (GGCACATGGACGAAATCTTGGTACTCT TCAGAATTC(T)24) and Taq polymerase (Amplitaq LD [ALD] or Amplitaq Stoffel Fragment [ASF]; Perkin Elmer). Samples shown in Figure 1 were prepared with: 1–8: AL1 plus ALD; 9–15: AL3 plus ALD; 16–23: AL1 plus ASF; 24–30: AL3 plus ASF.

Aliquots of each cDNA sample were electrophoresed on agarose gels and blotted onto nylon membranes (Hybond N^+, Amersham) (Ausubel et al., 1988; Sambrook et al., 1989). The blots were hybridized at 55°C or 70°C in Hyb buffer (0.5 M sodium phosphate buffer [pH 7.3], 4% SDS, 1% bovine serum albumin [BSA]) with ^{32}P-labeled probes prepared by random priming (Prime-It II, Stratagene).

Construction and Screening of Single-Cell cDNA Libraries

An aliquot of cDNA sample VN14 was digested with EcoRI, and gel-isolated fragments of 0.3 to 1.5 kb were cloned into λZapII (Ausubel et al., 1988; Sambrook et al., 1989). Two thousand library clones were plated at low density. Replica filter lifts were hybridized at 75°C (in Hyb buffer containing 2 μg/ml poly(dT)24 and 1 μg/ml of random dA-dT 20-mers) to ^{32}P-labeled probes ($\sim 2.5 \times 10^8$ cpm/μg; 5×10^6 cpm/ml) prepared by PCR of different single-cell cDNA samples. Clones that hybridized to only a VN14 probe were isolated, and a probe prepared from the insert of each was hybridized to blots of selected single-cell cDNAs. Clones that hybridized to only VN14 cDNAs were sequenced.

Isolation and Analysis of VR cDNA Clones

sc153, one $VN14^+VN2^-$ clone from the VN14 library, was used as probe to screen a mouse VNO cDNA library (λVNO) (Berghard and Buck, 1996) and a mouse genomic DNA library (Stratagene) (70°C, Hyb buffer). Hybridizing clones were found only in the genomic library. A fragment containing 2 kb upstream of sc153 was isolated from one genomic clone (153G1) and used to screen λVNO (55°C, Hyb buffer). The region (D10-TM7) of one clone (D10) that showed homology to TM7 of the CSR (see Results) was then used to screen λVNO (55°C, Hyb buffer), yielding a variety of VR cDNA clones. Additional clones were obtained from λVNO using probes prepared from clones previously isolated or from PCR products obtained by amplification of mouse genomic DNA or VNO cDNA with degenerate primers (Buck and Axel, 1991) matching conserved motifs in the VRs. Some PCR products were also cloned into pCR2.1 (Invitrogen) and sequenced.

Analysis of VR mRNAs by RT-PCR

Random-primed cDNA prepared from male or female C57BL/6J mouse VNO RNAs (or VR cDNA clones) were used in PCR reactions with degenerate primers (Buck and Axel, 1991) matching conserved VR motifs to amplify VR sequences corresponding to amino acids 33 to 772 in VR1. Nested PCR was performed with a 1/1000 dilution of the first PCR reaction and primer pairs matching regions of putative exons 1 and 6 in specific VR cDNA clones. Blots prepared from size-fractionated, nested PCR products were hybridized (70°C, Hyb buffer containing 100 μg/ml herring sperm DNA [Sigma]) to probes prepared from the PCR products of the cDNA clones.

Northern and Southern Blots and Genomic Library Screens

Northern blots: 1 μg of $Poly(A)^+$ RNA prepared from mouse VNO and OE or purchased from Clontech (other tissue RNAs) was size fractionated on formaldehyde gels and blotted (see above) (Berghard and Buck, 1996). The blot was hybridized (70°C, Hyb buffer) with a ^{32}P-labeled probe prepared from the regions of cDNAs VR1, VR2, VR4, and VR15 corresponding to that encoding amino acids 33 to 772 in VR1.

Southern blots: 5 μg of genomic DNA prepared from C57BL/6J mouse liver was digested with EcoRI or HindIII, size fractionated, and blotted (Ressler et al., 1993). The blots were hybridized (70°C, Hyb buffer containing sperm DNA [see above]) to probes prepared from 3′ untranslated segments of different VR cDNA clones (VR2 [nt 2607–2961], VR3 [nt 2505–2907], and VR15 [nt 3239–3689]). A VR4 probe was also used, which gave the same results as a highly related VR15 probe.

Genomic library screens to determine VR gene number: a mouse genomic library was screened separately at 70°C or 55°C (see above) with different ^{32}P-labeled probes. Probe 1: a mix of segments of cDNAs VR1, VR2, VR4, and VR15 encoding the region corresponding to amino acids 619 to 772 of VR1. Probes 2–6: segments of VR

genes obtained from mouse genomic DNA by PCR with degenerate primers matching conserved VR sequence motifs. The PCR segments corresponded to the following amino stretches in VR1: 191–224, 565–825, 637–825, 637–804, and 619–784.

In Situ Hybridization

In situ hybridization was performed according to Schaeren-Wiemers and Gerfin-Moser (1993) with sequential 16 μm sections of male or female VNOs. Digoxigenin-labeled cRNA probes were prepared from the same 3′ untranslated regions of VR cDNAs as used for the genomic Southern blots. Sections were counterstained with Hoechst 33258, which labels nuclei. The number of $G\alpha_o$- or $G\alpha_{i2}$-labeled cells (or cells labeled with VR probes) was determined by counting the number of nuclei in labeled regions. The total number of cells was considered to be the sum of $G\alpha_o^+$ and $G\alpha_{i2}^+$ cells in adjacent sections.

Chromosome Mapping of VR Genes

Southern blots of genomic DNA from C57BL/6J and Mus spretus (Jackson Labs) digested with different restriction enzymes were prepared and probed with specific VR cDNA probes as described above. Southern blots of EcoRI-digested, size-fractionated genomic DNAs from 94 different backcross mice (M. spretus × (M. spretus × C57BL/6J)) were purchased from Jackson Labs. These blots were hybridized to probes prepared from 3′ untranslated segments of the VR2 or VR4 (see above) cDNA at 70°C and washed (see above). Polymorphic bands were typed as either M. spretus or M. spretus/C57BL/6J. The data was sent to the Jackson Laboratory Backcross DNA Mapping Panel Resource for determination of the chromosomal locations of the polymorphic fragments. Additional information was obtained via internet from Jackson Laboratory Mouse Genome Informatics.

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